Spectrophotometric Estimation of Protein Concentration in the Presence of Tryptophan Modified by 2-Hydroxy-5-nitrobenzyl Bromide

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A spectrophotometric method makes it possible to determine the concentration of a protein after covalent modification of tryptophan residues by 2-hydroxy-5-nitrobenzyl bromide. Molar absorption coefficients for the 2-hydroxy-5-nitrobenzyl chromophore, reported here in the pH range from 4.0 to 10.9, can be used to correct the protein absorbance values at 280 nm, which then provides the basis for calculating protein concentration in the usual way. The method was tested with α-lactalbumin, β-lactoglobulin, pepsin, and soybean trypsin inhibitor; spectrophotometrically estimated concentrations of these proteins agreed closely with values obtained by amino acid analysis.

KEY WORDS: protein concentration; 2-hydroxy-5-nitrobenzyl bromide; tryptophan; α-lactalbumin; β-lactoglobulin; pepsin; soybean trypsin inhibitor.

Koshland's reagent, 2-hydroxy-5-nitrobenzyl bromide (HNB),[2] has been a desirable choice for modifying tryptophan residues in proteins since it was first described (1). HNB reacts rapidly and specifically at position 3 of the indole ring of tryptophan (2,3), is nondestructive, and has the excellent advantage of rendering each modified tryptophan residue nonfluorescent. For a protein having more than one tryptophan residue this means that the fluorescence of tryptophans not accessible to HNB is the only emission observed after modification (4–6). Furthermore, if all tryptophans react with HNB it may be possible to observe the emission of tyrosine, which is usually not observed in proteins containing both tyrosine and tryptophan (7).

For such fluorescence studies protein concentration must be carefully controlled, as high absorbance values produce inner-filter effects (8) needing substantial correction, and these could compromise the validity of emission results. Moreover, for comparing the relative quantum yields of HNB-modified and unmodified protein samples, their spectra must be adjusted to represent identical protein concentrations. Thus, accurate estimates of protein concentration are needed.

Determining the concentration of an HNB-modified protein is not straightforward, however, because the pH-dependent spectrum of the HNB chromophore (Fig. 1) affects the protein spectrum and thus interferes with spectrophotometric determination of protein concentration using known molar absorption coefficients (ε). Other protein assay methods, including microbiuret (9), Coomassie blue binding (10), and Lowry (11), cannot always substitute for determining the concentration of HNB-modified proteins (see Discussion). Amino acid analysis after HCl hydrolysis is a good alternative for determining protein concentration, but sample preparation and

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[2] Abbreviations used: BSA, bovine serum albumin; BGG, bovine gamma globulin; HNB, 2-hydroxy-5-nitrobenzyl bromide or 2-hydroxy-5-nitrobenzyl group; HNBOH, 2-hydroxy-5-nitrobenzyl alcohol; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; MES, mercaptoethanesulfonic acid; STI, soybean trypsin inhibitor.

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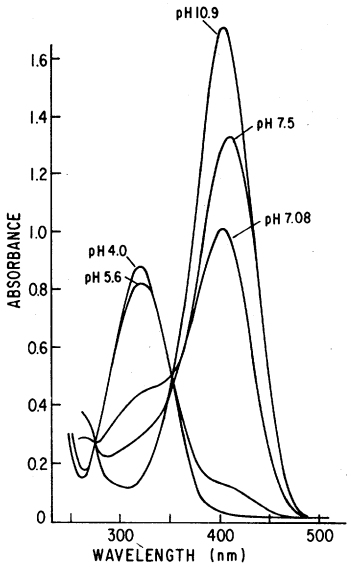

FIG. 1. Effect of pH on the ultraviolet–visible spectrum of HNBOH. Spectral properties of the HNB chromophore in HNBOH and in an HNB-modified protein are similar (1).

analysis may take as much as 3 days and access to amino acid analysis is not available in every laboratory.

In the course of studies probing the effect of pH on buried and exposed tryptophan residues in β-lactoglobulin (β-Lg), we have developed a spectrophotometric procedure for obtaining accurate protein concentrations in the presence of HNB-tryptophan residues. Since the spectral properties of the HNB chromophore in HNBOH and in a protein covalently modified by HNB are similar (1), the pH-dependent molar absorption coefficients of HNBOH are used to correct protein absorbance values at 280 nm. With this procedure experimental work can continue almost immediately when protein concentration must be known accurately, as for fluorescence studies, other physical measurements, and assays for enzyme activity. The actual number of tryptophans modified can be determined later by amino acid analysis of mercaptoethanesulfonic acid (MES) hydrolysates or by comparing the quantum yields of normalized protein emission spectra before and after modification.

MATERIALS AND METHODS

HNB was a product of Sigma Chemical Company.[3] All other reagents used were reagent grade or better. Acetone (Mallinckrodt Nanograde) was dried over anhydrous magnesium sulfate before use. β-Lg was prepared from bovine milk according to the procedure of Aschaffenburg and Drewry (12). Pepsin was a product of Worthington Biochemical, and soybean trypsin inhibitor (STI, Kunitz type) was from Sigma. α-Lactalbumin (α-La) was prepared as described (12).

A Radiometer pH meter equipped with a combination electrode was used for pH determinations, and ultraviolet–visible spectra were obtained at each pH with a Cary 14 or Beckman Model 25 spectrophotometer in black-masked semimicro quartz cuvettes of 1-cm pathlength. A JEOL 60 MHz spectrometer was used for the NMR spectrum of HNBOH in deuterated methanol (CD_3OD). Amino acid analyses of MES and HCl hydrolysates of proteins previously modified with HNB were obtained with either Phoenix or Beckman 119CL analyzers. Protein concentrations from integrated amino acid analyses were based on the content of a stable amino acid in the sample (usually lysine, arginine, or phenylalanine) and the known amino acid composition of the protein.

Determination of molar absorption coefficients. HNBOH was prepared by slowly adding 200 mg HNB in a minimum amount of dry acetone (<100 μl) to 1.5 ml deionized water while stirring. The resulting white precipitate was dissolved by addition of 95% ethanol, and the solution was evaporated to dryness with a rotary evaporator. The solid was redissolved in 100 ml 95% ethanol for the stock solution. To assess the purity of

[3] Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

the stock material, an aliquot was evaporated and dissolved in CD_3OD, and an NMR spectrum was obtained. Peaks corresponded to those expected for HNBOH plus traces of ethanol and methanol (the latter from contamination in CD_3OD). No evidence was found of a rearranged compound, 2-hydroxy-5-nitroanisole, previously reported to be an additional reaction product (3).

To prepare solutions for determining molar absorption coefficients, 0.1-ml aliquots of the stock HNBOH solution were accurately pipetted onto weighing dishes, air-dried, and transferred to a vacuum desiccator for 2 h. Each dried sample was dissolved, transferred to a 10-ml volumetric flask, adjusted to volume with one of a series of solutions in the pH range 4.0 to 10.9, and mixed thoroughly. The pH of each solution was measured after mixing, and an ultraviolet–visible spectrum was obtained. The reproducibility of this technique was demonstrated by spectral measurements of 1.0 ml of each solution diluted to 3.0 ml with 6 N NaOH. This increased the pH of each solution to 11.0, where maximum absorption is observed (Fig. 1). Absorbance values at 410 nm agreed within 0.3%.

The concentration of the pH 10.9 HNBOH solution was calculated to be 9.33×10^{-5} M from its absorbance at 410 nm using an average of the molar absorption coefficients reported previously (1,13), 18,400 M^{-1} cm^{-1}. The spectra of other HNBOH solutions were adjusted to correspond to concentrations identical to that of the pH 10.9 solution; correction factors were the ratios of the 410-nm absorbance values of each solution diluted in 6 N NaOH to that of the diluted pH 10.9 solution.

Protein modification. Proteins were modified by HNB in buffers appropriate for the desired pH values using techniques described previously (4,5). Briefly, an approximately 25-fold excess of HNB over the concentration of tryptophan present in a protein solution (5 to 10 mg protein/ml) was added in a small amount (<5% v/v) of anhydrous acetone. After it was stirred 20 min at room temperature the solution was centrifuged in a clinical centrifuge (RCF = 275), eluted from a column of Sephadex G-25, and dialyzed vs the same buffer with at least four changes or until the dialysate was no longer yellow. After dialysis, spectra were obtained of the dialyzed protein (450 to 240 nm) and of a 5- or 10-fold dilution of the HNB-protein with 2 N NaOH (550 to 375 nm).

RESULTS

The ultraviolet–visible absorption spectra of HNBOH solutions (Fig. 1) show the effect of pH on the wavelength and magnitude of the absorption peaks. The variation in wavelength maximum for HNBOH is due to ionization of the 2-hydroxy group on the aromatic ring, with the unionized form absorbing at 320 nm and the ionized form at 410 nm. At pH 7.5 the trailing edge of the nitrophenol chromophore on the lower-wavelength side of the 320-nm peak begins to influence the 280-nm absorption; this effect increases as pH decreases. At higher pH values the effect of the HNB group at 280 nm probably arises only from the contribution of the substituted phenyl ring and is much less pronounced.

The molar absorption coefficient values in Table 1 and Fig. 2 are used to correct the apparent protein absorbance at 280 nm after gel chromatography and dialysis (Eq. [1]):

TABLE 1

MOLAR ABSORPTION COEFFICIENTS OF 2-HYDROXY-5-NITROBENZYL ALCOHOL (HNBOH)

	ϵ (M^{-1} cm^{-1} × 10^{-3})		
pH	280 nm	320 nm	410 nm
4.00	3.49	9.38	—
4.96	3.77	—	—
5.10	3.80	9.06	0.664
5.60	3.95	8.99	1.37
7.08	2.80	4.72	10.9
7.50	2.48	3.23	14.7
8.90	2.15	1.69	17.7
10.9	1.89	1.40	18.4[a]

[a] Average of literature values (1,13).

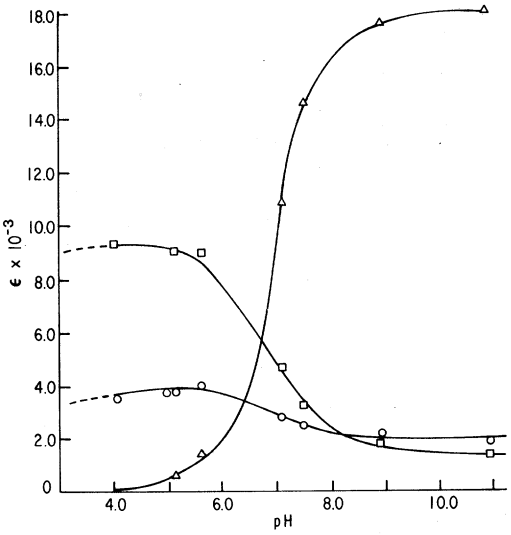

FIG. 2. Effect of pH on molar absorption coefficients for HNBOH. Data points from Table 1. – – –, Extrapolated to pH 3.0; ○, 280 nm; □, 320 nm; △, 410 nm.

$$A_{280} = (C_{prot}) \times (\epsilon_{prot}) + (C_{HNB}) \times (\epsilon_{HNB}) \quad [1]$$

where C_{prot} represents the unknown molar protein concentration, ϵ_{prot} is the molar absorption coefficient of the unmodified protein at 280 nm, C_{HNB} is the molar concentration of HNB, and ϵ_{HNB} a molar absorption coefficient at 280 nm from Table 1 or Fig. 2. C_{HNB} is obtained by diluting an aliquot of the HNB-modified protein with 2 N NaOH, which increases the pH to 11 or more where the nitrophenolate form of the chromophore predominates. The average molar absorption coefficient at 410 nm, 18,400 M^{-1} cm^{-1}, is used to calculate the concentration of HNB chromophore used in Eq. [1] after adjusting for dilution. With ϵ_{HNB} and ϵ_{prot} at 280 nm known, the protein concentration is easily calculated. Table 2 compares concentrations of HNB-proteins obtained using the spectrophotometric method with values calculated from amino acid analyses as described (Materials and Methods). Close agreement shows that spectrophotometric estimates of HNB-protein concentrations can be used with confidence.

The ultraviolet–visible spectrum of HNB-STI (see Table 2) shown in Fig. 3 is typical of many HNB-modified proteins. The spectrum of the HNB chromophore, which lies underneath that of the intrinsic protein between 250 and 320 nm, was calculated (at pH 5.2) using Table 1 and Fig. 2 and is shown on the figure to indicate the extent of error introduced by the HNB chromophore. The points represent the addition of spectra of unmodified STI and of HNB chromophore. Both STI and HNB concentrations are the same as in the HNB-STI sample in Table 2. The superposition of the addition spectrum with that of the HNB-STI shows that the intrinsic protein spectrum is not affected by covalent HNB modification and that Eq. [1] has a sound theoretical basis.

Because the wavelength maximum of the HNB chromophoric group is near the wavelength maximum of the protein at pH levels below 7.0 the spectrum of an HNB-protein can be distorted, especially if the protein contains a number of reactive tryptophan residues. Table 2 shows that the concentration can still be calculated correctly down to pH 4.4. At pH 3.0, however, estimated concentrations are lower than amino acid analysis results because the ϵ_{280} of proteins decreases in acidic solutions. However, the overlap of

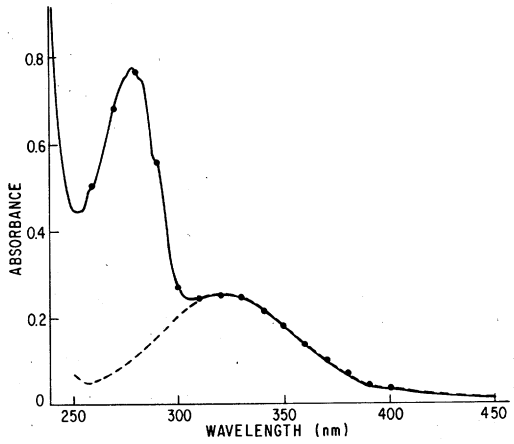

FIG. 3. HNB-modified STI. ——, Spectrum of the modified protein; – – –, spectrum of the HNB chromophore, calculated as described in the text, shows how it displaces that of the protein; ●, addition spectrum of unmodified STI and HNB chromophore, each at concentrations of the HNB-STI sample in Table 2.

TABLE 2

COMPARISON OF PROTEIN CONCENTRATIONS OBTAINED WITH SPECTROPHOTOMETRIC METHOD AND AMINO ACID ANALYSIS RESULTS

Protein (number of Trp)	pH		HNB ϵ_{280} (M^{-1} cm^{-1})	Protein concentration (mg/ml)	
	HNB modification	A_{280} for Eq. [1]		Spectrophotometrically determined	Calculated from amino acid analysis
HNB-β-Lg (2)	9.0	9.0	2150[a]	1.28	1.28
	7.5	7.5	2480[a]	1.16	1.17
	4.65	7.5	2480[a]	2.16	2.18
	4.4	4.4	3700[b]	2.66	2.82
	3.0	7.0	2800[a]	1.04	1.08
	3.0	3.0	3200[b]	2.26[c]	2.76
HNB-α-La (4)	6.00	7.75	2200[b]	0.195	0.204
HNB-pepsin (5)	6.00	6.00	3700[b]	1.10	1.03
HNB-STI (2)	5.3	5.2	3800[b]	0.687	0.678

[a] From Table 1.
[b] From Fig. 2.
[c] See text for discussion of accuracy below pH 4.4.

protein and HNB bands and the effects of acidic pH can be eliminated by adding, to an aliquot of a sample modified at low pH, sufficient base to increase the pH to between 7 and 8. This produces a shift in the spectrum of the HNB chromophore and "frees" the protein absorption band for a more accurate reading. The protein concentration can then be calculated as before after correction for dilution. The success of this approach is demonstrated by results for HNB-α-La and for HNB-β-Lg which had been modified at pH 3.0 but estimated at pH 7.0 (Table 2).

DISCUSSION

Although tryptophan is highly hydrophobic and therefore likely to be found in the interior portions of globular proteins, its indole ring also has polar character at the indole nitrogen and indeed occurs exposed to solvent in some proteins. An example is tryptophan-15 located near the amino terminus of horse liver alcohol dehydrogenase (4,5,14). The presence of both buried and exposed tryptophan residues in the same protein provides ideal conditions for conducting fluorescence energy transfer and quenching studies to aid in mapping active sites and molecular distances. In native proteins, exposed tryptophan residues react readily with HNB whereas buried residues cannot, since competitive reaction of solvent water with HNB occurs much more rapidly than entry of unreacted HNB into interior regions, the latter being highly dependent on breathing rates of protein domains (15). HNB is therefore a useful probe for studying buried vs exposed tryptophan residues.

However, difficulties have been encountered in the use of HNB in the past. A primary problem is that each tryptophan residue can become doubly modified by the addition of a second HNB group[4] at the indole ring nitrogen or at position 2 of the ring (4,5,16). The extent of this additional reaction with HNB is largely influenced by its microenvironment, and this leads to variability in the number of HNB groups covalently bound to each tryptophan residue. For this reason, the use of HNB to determine the

[4] After this work was completed, the problem of double labeling was discussed and the existence of multiple diastereomeric forms of HNB-labeled tryptophan in a peptide reported (18).

tryptophan content of a protein of unknown composition after denaturation, as once proposed (17), does not produce accurate results. Noncovalent binding of HNBOH is the second problem that arises. Karkhanis (13) has suggested a modified procedure which is somewhat helpful in overcoming both double labeling and noncovalent binding of HNBOH, but it also involves protein denaturation and is inappropriate when HNB is being used to compare protein domains by probing for exposed and buried tryptophans.

Gel chromatography and dialysis after protein modification will eliminate both unreacted reagent and noncovalently bound HNBOH, but the interference of HNB at 280 nm still affects determination of protein concentration. The method described here provides accurate data and requires only two absorbance measurements. In contrast, amino acid analysis is not always available, and other assay methods are not reliable. The HNB chromophore interferes with a very accurate version of the microbiuret method in which assay samples are read at 300 nm (9). Furthermore, the Coomassie blue dye binding assay (10) gives variable results with many proteins (as the manufacturer has noted), especially those which have fewer available positive charges than the commonly used protein standards, bovine serum albumin (BSA) and bovine gamma globulin (BGG). For example, β-Lg has a Coomassie blue assay slope of 0.1 ΔA ml mg^{-1} compared with a value of 0.6 ΔA ml mg^{-1} for BGG and for reduced and carboxymethylated β-Lg.[5] The Lowry assay (11) requires preparation of a standard curve for each protein because of its nonlinearity and is subject to interferences by a number of chemical groups.

The spectrophotometric method for estimating protein concentration after modification enhances the utility of HNB as a probe of tryptophan residues and their environments because it enables experimental work to continue after a minimal delay. Rapid dialysis equipment might reduce post-modification clean-up time further. With the simple procedure described, plus Eq. [1] and an appropriate value from Table 1 or Fig. 2, the concentration of HNB-modified protein can be readily calculated.

[5] E. L. Malin, unpublished observation.

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